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Review article

# Metabolic engineering for the production of plant isoquinoline alkaloids

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## Summary

Several plant isoquinoline alkaloids (PIAs) possess powerful pharmaceutical and biotechnological properties. Thus, PIA metabolism and its fascinating molecules, including morphine, colchicine and galanthamine, have attracted the attention of both the industry and researchers involved in plant science, biochemistry, chemical bioengineering and medicine. Currently, access and availability of high-value PIAs [commercialized (e.g. galanthamine) or not (e.g. narciclasine)] is limited by low concentration in nature, lack of cultivation or geographic access, seasonal production and risk of overharvesting wild plant species. Nevertheless, most commercial PIAs are still extracted from plant sources. Efforts to improve the production of PIA have largely been impaired by the lack of knowledge on PIA metabolism. With the development and integration of next-generation sequencing technologies, high-throughput proteomics and metabolomics analyses and bioinformatics, systems biology was used to unravel metabolic pathways allowing the use of metabolic engineering and synthetic biology approaches to increase production of valuable PIAs. Metabolic engineering provides opportunity to overcome issues related to restricted availability, diversification and productivity of plant alkaloids. Engineered plant, plant cells and microbial cell cultures can act as biofactories by offering their metabolic machinery for the purpose of optimizing the conditions and increasing the productivity of a specific alkaloid. In this article, is presented an update on the production of PIA in engineered plant, plant cell cultures and heterologous micro-organisms.



## Introduction

Plants are major producer of structurally diverse and medicinally important alkaloid specialized metabolites that have great economical importance. There are over 21 000 known alkaloids that have been extracted from plants (Wink, 2010). Throughout history, alkaloid-producing plants, and their extracts, have been exploited for medicinal, ceremonial and toxic properties, leading up to knowledge that inspired today's wide range of industrial applications. Major commercial alkaloids include the analgesic morphine from opium poppy (*Papaver somniferum*), the acetylcholinesterase inhibitor galanthamine from daffodils (*Narcissus pseudonarcissus*), the antimicrobial sanguinarine from California poppy (*Eschscholzia californica*), the gout and anti-inflammatory drug colchicine from meadow saffron (*Colchicum autumnale*) and the metabolic precursor reticuline used for semi-synthetic alkaloid manufacture. All of these examples are alkaloids that belong to the family of plant isoquinoline alkaloids (PIAs) which includes high-value metabolites such as emetine, kreysigine, lycorine, magnoflorine, narciclasine, protopine and scoulerine to name a few (Figure 1).

## Isoquinoline alkaloids in plant

Pharmaceutical and commercial alkaloids can be obtained directly from plant source or are chemically converted after isolation. However, access and availability of some high-value PIAs (e.g. galanthamine and narciclasine) may be limited by low abundance in nature, lack of cultivation or geographic access, seasonal production and risk of overharvesting wild plant species. Often, their production via field cultivation, if possible, leads to supply inefficiencies and variability. For example, the yield of morphine from opium poppy extracts varies greatly due to the cultivar used, growing season, climate, etc. (Desgagne-Penix *et al.*, 2012; Dittbrenner *et al.*, 2009). In most cases, the availability of the PIA-producing plants is limited and/or difficult to farm. In addition to the low concentration, the complex mixtures of alkaloid *in planta* limit isolation and purification of PIA using conventional extraction processes, especially those intended for commercial use. Alternative production systems, such as total or semi-synthesis of PIAs, have been developed but are typically complex for a low yield of isoquinoline alkaloid (Banwell *et al.*, 2012; Graening and Schmalz, 2004; Pulka, 2010; Rinner and Hudlicky,

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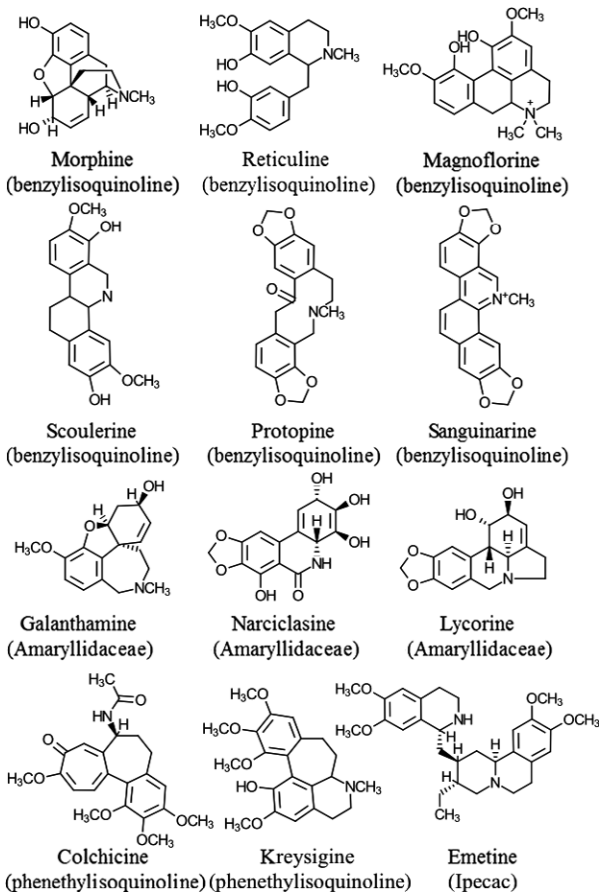


**Figure 1** Examples of plant isoquinoline alkaloids (PIAs) found in plants. The structural group or the plant family to which each alkaloid belongs is indicated in parentheses.

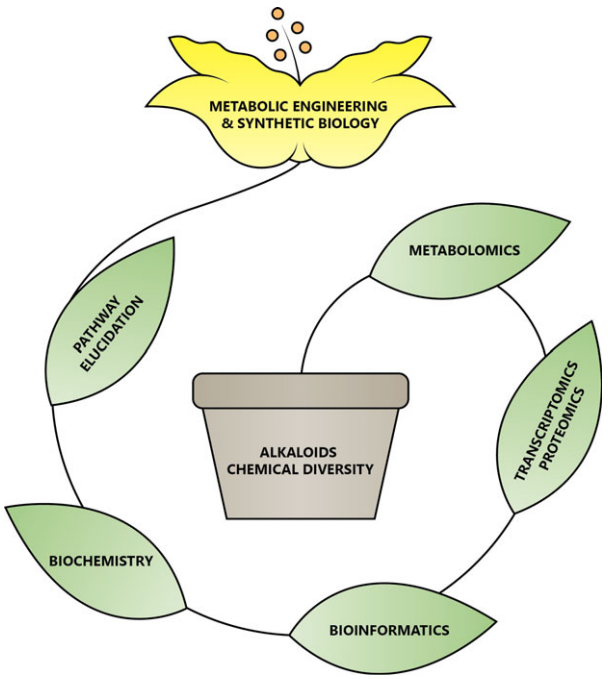


**Figure 2** Overview of the systems biology strategy feeding into metabolic engineering and synthetic biology approaches. Plants produce several thousand of different alkaloid metabolites; several of which are used by humans as high-value pharmaceuticals and precursors for various chemicals and bioproducts. Multidisciplinary systems biology strategy offers new avenues to explore and exploit this natural resource. Tissue-specific identification of known and novel compounds through metabolite profiling or targeted metabolomics informs genomics-based (transcriptomics and proteomics) discovery and elucidation of alkaloid-biosynthetic genes, enzymes and pathways via bioinformatics and biochemistry approaches. The elucidated pathways provide tools for combinatorial plant and microbial metabolic engineering and synthetic biology biotechnologies to be developed for plant-based bioproducts.

2012). Consequently, there is a demand not only for better isolation and production platforms but also for alternative ways to produce the valuable alkaloids using biotechnological processes such as plant engineering, plant cell culture or microbial metabolic engineering. Each strategy has its own challenges, and the most efficient and economically viable one will largely depends on the metabolite produce and may change over time due to technological innovations, increase in knowledge and economic and political developments (Keasling, 2012).

## Biosynthesis of isoquinoline alkaloids

Most PIAs accumulate at low concentration levels in plants. For example, analysis of galanthamine content in Amaryllidaceae plants showed variation from trace to 0.6% (referred to DW) with the commercial cultivar *Narcissus confusus* being the most productive of this family (Berkov *et al.*, 2009). Also, PIAs are metabolites of limited taxonomic distribution and often represent signature molecules of certain plant species or families. For example, the benzylisoquinoline alkaloid morphine and codeine are only produced in the *P. somniferum* plant species. Similarly, the Amaryllidaceae alkaloids, such as galanthamine, antimicrobial lycorine and anticancer narciclasine, are only synthesized in plants of the Amaryllidaceae family (e.g. amaryllis, daffodils and snowdrops). Biosynthesis and accumulation of specialized isoquinoline alkaloids can be limited to specific cells types, tissues or organs, and may be regulated in response to environmental perturbations (Beaudoin and Facchini, 2014; Desgagné-Penix and Facchini, 2011; Onoyovwe *et al.*, 2013). In an ecological context, PIAs have been suggested to be involved in plant defence mechanisms owing to their physiological activity, either by direct toxic effect (deterrent) or by inhibition of certain functions of the invading pathogens (Berkov *et al.*, 2008; Gomez *et al.*, 2003; Nomura *et al.*, 2008; Santana *et al.*, 2008; Shen *et al.*, 2014; Shields *et al.*, 2008; Simas *et al.*, 2001).

Advances in understanding PIA metabolism were originally achieved through isotopic tracer studies, enzyme isolation and characterization methods, and recombinant DNA technologies (Cordell, 2013). Rapid progress in plant systems biology, which combines metabolite profiling, transcriptome sequencing, bioinformatics and biochemistry, has accelerated gene discovery across a diversity of plant species including the alkaloid-producing plants where genomic information is often unavailable (Figure 2) (Bohlin *et al.*, 2010; Facchini *et al.*, 2012; Higashi and Saito, 2013; Schilmiller *et al.*, 2012; Seaver *et al.*, 2014; Sheth and Thaker, 2014; Trewavas, 2006; Wolfender *et al.*, 2013; Xiao *et al.*, 2013). For PIA-producing plants, RNA sequencing has been used to generate transcriptome databases of more than 30 plant species, mostly benzylisoquinoline alkaloids producing ones (Desgagne-Penix *et al.*, 2010, 2012; Farrow *et al.*, 2012; Hagel *et al.*, 2015;

Kilgore *et al.*, 2014; Wang *et al.*, 2013; Xiao *et al.*, 2013). These have allowed for the identification of several biosynthetic genes and provided knowledge for understanding PIA metabolism. Thus, the systems biology approach has not only accelerated the elucidation of the PIA pathways but has also increased the number of gene sequences (i.e. enzyme variants or tools) to be used in engineering production systems.

Plant isoquinoline alkaloids are synthesized from tyrosine decarboxylated precursor (tyramine or dopamine) which is condensed with a second precursor to form a specific scaffold molecule (norcoclaurine, autumnaline, deacetylisoipecoside or norbelladine) (Figure 3). Further modifications and decorations of the scaffold molecule lead to the wide array of PIAs known to date (Figures 1 and 3). Four groups of PIAs are presented here, namely the benzylisoquinoline, phenethylisoquinoline, ipecac and Amaryllidaceae alkaloids (Figure 3). The most studied metabolic pathways belong to the benzylisoquinoline group of PIA, specifically *P. somniferum* morphinan (e.g. morphine/codeine), *E. californica* protoberberine (e.g. scoulerine) and benzophenanthridine (e.g. sanguinarine) and *Coptis japonica* aporphine (e.g. magnoflorine) biosynthetic pathways. Consequently, these have emerged as model systems to study and engineered PIA metabolism. To date, over 30 biosynthetic genes have been reported to be involved in benzylisoquinoline alkaloid pathways (Beaudoin and Facchini, 2014; Desgagné-Penix and Facchini, 2011; Hagel and Facchini, 2013). The knowledge acquired on these pathways showed that it involves a restricted number of enzyme families catalysing coupling reactions and functional group modifications including Pictet-Spenglerases, cytochrome P450, acetyl-, *O*- and *N*-methyltransferases, oxidoreductases and dioxygenases (Hagel and Facchini, 2013;). To date, only five biosynthetic genes for ipecac alkaloids, one for Amaryllidaceae alkaloids and none for the phenylethylisoquinoline alkaloids have been reported (Figure 3) (Cheong *et al.*, 2011; Kilgore *et al.*, 2014; Nomura and Kutchan, 2010; Nomura *et al.*, 2008). As metabolic engineering requires knowledge of the pathway genes and enzymes involved, the majority of the studies focus on the benzylisoquinoline alkaloid pathways.

## Plant metabolic engineering and plant cell cultures

When the cultivation of PIA-producing plant is difficult or not practically possible, production in plant cell cultures is economically feasible for certain compounds, particularly those of high value. Amaryllidaceae *Leucojum aestivum* and *Pancratium maritimum* plant cell cultures have been developed for the study and production of galanthamine and lycorine (Bogdanova *et al.*, 2009; Georgiev *et al.*, 2010; Ptak *et al.*, 2010; Saliba *et al.*, 2015). Similarly, cell cultures of 18 plant species that produce benzylisoquinoline alkaloids have been developed and used to study and identify genes involved in PIA metabolism (Desgagne-Penix *et al.*, 2010; Farrow *et al.*, 2012). The basal production of PIAs in these cell culture systems is low, and increased production requires costly addition of metabolic precursor, plant hormone or fungal elicitor (Cho *et al.*, 2008; Desgagne-Penix *et al.*, 2010; Hara *et al.*, 1993; Ivanov *et al.*, 2012; Nakagawa *et al.*, 1986; Pavlov *et al.*, 2007; Verma *et al.*, 2014). For example, untreated opium poppy cells do not produce alkaloids, whereas elicitor-treated cells showed up-regulated expression of biosynthetic enzymes and increased production of sanguinarine but not morphine (Desgagne-Penix *et al.*, 2010). In plants, there is a clear

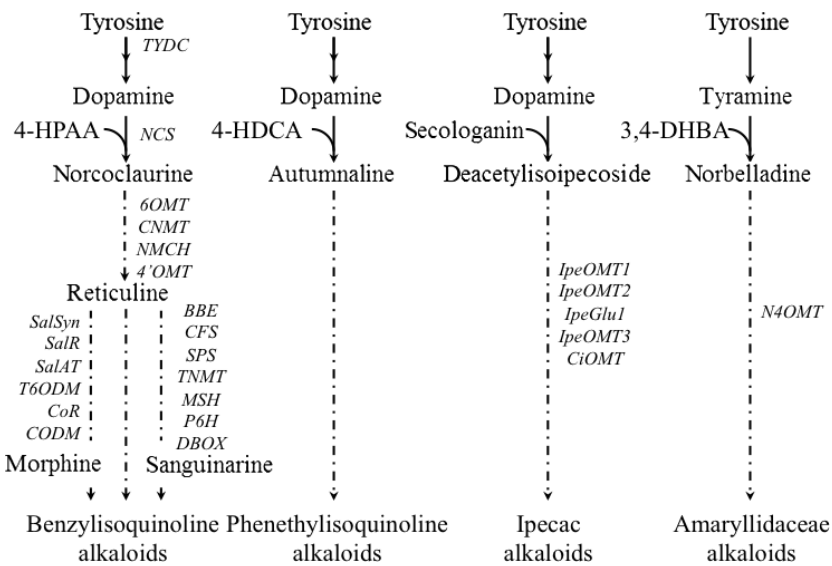


**Figure 3** Overview of the biosynthetic pathways of four groups of plant isoquinoline alkaloids including the benzylisoquinoline, phenethylisoquinoline, Ipecac and Amaryllidaceae alkaloids. Enzymes for which corresponding genes have been isolated from plants are shown in *italic*. Bold arrow represents one biochemical reaction, whereas dotted arrows represent more than one reaction. 4-HPAA, 4-hydroxyphenylacetaldehyde; 4-HDCA, 4-hydroxydihydrocinnamaldehyde; 3,4-DHBA, 3,4-dihydroxybenzaldehyde; *TYDC*, tyrosine/DOPA decarboxylase; *NCS*, norcoclaurine synthase; *6OMT*, norcoclaurine 6-*O*-methyltransferase; *CNMT*, coclaurine *N*-methyltransferase; *NMCH*, *N*-methylcoclaurine; *4′OMT*, 3′-hydroxyl-*N*-methylcoclaurine 4′-*O*-methyltransferase; *SalSyn*, salutaridine synthase; *SalR*, salutaridine reductase; *SalAT*, salutaridinol 7-*O*-acetyltransferase; *T6ODM*, thebaine 6-*O*-demethylase; *CoR*, codeinone reductase; *CODM*, codeine *O*-demethylase; *BBE*, berberine bridge enzyme; *CFS*, cheilanthifoline synthase; *SPS*, stylopine synthase; *TNMT*, tetrahydroprotoberberine *N*-methyltransferase; *MSH*, *N*-methylstylopine 14-hydroxylase; *P6H*, protopine 6-hydroxylase; *DBOX*, dihydrosanguinarine oxidase; *IpeOMT1*,2,3, Ipecac *O*-methyltransferase-1, -2, -3; *IpeGlu1*, Ipecac glycosidase-1; *CiOMT*, *Carapichea ipecacuanha O*-methyltransferase; *N4OMT*, norbelladine 4-*O*-methyltransferase.

 

correlation between cellular differentiation and specialized metabolism. The low or lack of productivity of alkaloids in plant cell cultures can be explained by insufficient level of cell differentiation (Desgagne-Penix *et al.*, 2010; Farrow *et al.*, 2012). For plant cell cultures, the transition from shake flask to bioreactor is complicated. As a result, PIAs are rarely produced commercially using plant cell cultures due to high cost of maintenance versus low yield obtained. However, a wide variety of bioprocessing strategies have been specifically designed for large-scale cultivation of plant cells. One success story is the commercial production of antibiotic berberine from *C. japonica* and *Thalictrum minus* plant cell cultures by Mitsui Chemicals, Inc. (Tokyo, Japan) (Wilson and Roberts, 2012). The optimization of the industrial production from *C. japonica* cells at a scale up to 4 $m^3$ resulted in high berberine yield of 3.5 g/L and a 3.5-fold change compared with traditional methods (Georgiev *et al.*, 2013; Matsubara *et al.*, 1989). Metabolic engineering for increased productivity could decrease production costs associated with existing commercial plant cell culture systems and make other plant cell culture systems commercially feasible.

Isotopic tracer studies were used to trace PIA metabolic pathways but not to quantify fluxes through these pathways. For the past decade, isotope-assisted flux analyses have provided researchers with powerful indicators of cell biochemistry and a deeper understanding of metabolic pathways (Allen *et al.*, 2009; Dieuaide-Noubhani *et al.*, 2007; Niklas *et al.*, 2010; O'Grady *et al.*, 2012; Wiechert, 2001; Zamboni, 2011). Due to the significant level of complexity, compartmentation and the synthesis of a wide array of metabolites, a key issue of PIA accumulation in engineered plants and cell cultures is the channelling of maximized flux towards a biosynthetic sink. Flux analysis measurements in combination with other systems biology methods will provide the information needed for a rational approach of plant (and plant cell culture) metabolic engineering in the future.

Efforts to improve yields of PIAs are often hindered by limitations in plant metabolic engineering due to the lack of genetic tools, long development cycles of plants and the complex interaction between primary and specialized metabolic pathways (Chae *et al.*, 2014; Glenn *et al.*, 2013). However, plant metabolic engineering has been used to modulate PIA composition and yield of morphinan alkaloids in the 'model plant' opium poppy (*P. somniferum*) with mixed results. For example, the overexpression of gene, *N*-methylcoclaurine-3′-hydroxylase (*NMCH*), salutaridine-7-*O*-acetyltransferase (*SalAT*) or codeinone reductase (*CoR*), each encoding biosynthetic enzyme involved in morphine production (Figure 3), resulted in increased morphinan alkaloids, whereas suppression of *CoR* did not alter alkaloid levels (Allen *et al.*, 2004, 2008; Frick *et al.*, 2007; Larkin *et al.*, 2007). Overexpression of Arabidopsis transcriptional regulators in opium poppy resulted in increased codeine accumulation associated with the up-regulation of several BIA biosynthetic enzymes (Apuya *et al.*, 2008).

## Microbial engineering for PIA biosynthesis

Metabolic engineering of alkaloid production may serve as alternative or complementary approach to chemical synthesis, plant cell culture or plant biomass extraction. This includes the concept of reconstruction of plant alkaloid pathways in heterologous host systems, as well as combinatorial approaches using combinations of enzymes from different species. Combination of enzymes has been successfully used to identify new gene functions or to replace missing enzymatic step for the production of alkaloids in micro-organisms. For example, the construction of pathway for reticuline production used a combination of bacterial monoamine oxidase and plant genes from *C. japonica* (Minami *et al.*, 2008). The reconstruction of PIA biosynthetic pathways in micro-organism hosts raises multiple engineering challenges due to the complex, branched, multistep architecture of biosynthetic pathways (Figure 3). A number of alkaloid engineering platforms have been described (Table 1). *Escherichia coli* and the baker yeast (*Saccharomyces cerevisiae*) are currently the preferred microbial hosts for PIA production due to their robustness, scalability and the broad repertoire of tools for DNA assembly (Keasling, 2012).

Microbes engineered to express genes encoding PIA biosynthetic enzymes provide a novel approach for the development of scalable manufacturing processes. The availability of 'large' numbers of PIA biosynthetic genes from opium poppy and related plants has facilitated the reconstitution of several pathways leading of the production of benzylisoquinoline alkaloids in *E. coli* and yeast. For example, production of (*S*)-reticuline in *E. coli* was achieved by adding dopamine to the culture medium, some of which was converted into 3,4-DHPAA precursor by heterologously expressed bacterial monoamine oxidase, and further metabolized by *C. japonica* NCS, 6OMT, CNMT and 4′OMT to yield 11 mg/L of reticuline (Minami *et al.*, 2008) (Table 1). The fine-tuning of the production method using this platform increased reticuline yield to 54 mg/L (Kim *et al.*, 2013). Recent modifications of this platform include *de novo* synthesis of dopamine precursor by two additional bacterial enzyme, therefore eliminating the use of supplements which facilitate the linking of PIA metabolism to *E. coli* primary metabolism and enable a fermentation (Nakagawa *et al.*, 2011) and benchtop production (Nakagawa *et al.*, 2012) platforms that create plant products from simple carbon sources (Table 1). Reticuline is a scaffold intermediate metabolite shared among major branches of the benzylisoquinoline pathway (Figure 3). Despite of high titres, no steps downstream of (*S*)-reticuline have since been demonstrated in *E. coli*. However, *E. coli*-generated reticuline was converted to alkaloids magnoflorine or scoulerine via coculture with genetically engineered *S. cerevisiae* expressing *C. japonica*-specific gene CYP80G2 and BBE, respectively (Minami *et al.*, 2008).

Alternatively, relatively high titre of (*R*,*S*)-reticuline (32.9 mg/L), tetrahydrocolumbamine (60 mg/L) and tetrahydroberberine (30 mg/L) was produced from *S. cerevisiae* from cost-prohibitive substrate (*R*,*S*)-norlaudanosoline through a combination of enzymes derived from different plant species and humans (Hawkins and Smolke, 2008). Recent modification of this (*R*,*S*)-reticuline platform allows the production of various PIAs such as stylopine, *N*-methylstylopine, protopine and sanguinarine (Trenchard and Smolke, 2015). Fossati *et al.* (2014) reconstituted a 10-gene plant pathway in *S. cerevisiae* that allows for the production of dihydrosanguinarine and sanguinarine from (*R*,*S*)-norlaudanosoline. Synthesis of dihydrosanguinarine also yields the side products *N*-methylscoulerine and *N*-methylcheilanthifoline, and the latter has not been detected in plants (Fossati *et al.*, 2014). Similarly, yeast strains modified with *P. somniferum* morphinan biosynthetic genes produced morphine and codeine when supplemented with costly intermediates such as thebaine, salutaridine or codeine (Fossati *et al.*, 2015; Thodey *et al.*, 2014) (Table 1).

 

**Table 1** Plant isoquinoline alkaloids (and precursors) produced by bioengineered micro-organisms

| Compound target | Source of plant gene inserted | Engineered host | Externally fed precursor | Titre (μg/L) | Yield (g product/g substrate) | References |
|---|---|---|---|---|---|---|
| (R-*S*)-reticuline | *Micrococcus luteus* (MAO) and *Coptis japonica* (NCS, 6OMT, CNMT and 4′OMT) | *Escherichia coli* | Dopamine | $11 \times 10^3$ | 0.029 | Minami *et al.* (2008) |
| (*S*)-reticuline | *M. luteus* (MAO) and *C. japonica* (NCS, 6OMT, 4OMT and CNMT) | *E. coli* | Dopamine | $54 \times 10^3$ | $0.237 \times 10^{-3}$ | Kim *et al.* (2013) |
| (*S*)-reticuline | *E. coli* (*tyrA*$^{fbr}$, *aroG*$^{fbr}$, *tktA and* ppsA), *C. japonica* (NCS, 6OMT, 4OMT and CNMT), *Pseudomonas putida* (DODC), *M. luteus* (MAO) and *Ralstonia solanacearum* (TYR) | *E. coli* | Glycerol | $46.0 \times 10^3$ | n.a. | Nakagawa *et al.* (2011) |
| (*S*)-reticuline | *E. coli* (*tyrA*$^{fbr}$, *aroG*$^{fbr}$, *tktA and* ppsA), *C. japonica* (NCS, 6OMT, 4OMT and CNMT), *P. putida* (DODC), *M. luteus* (MAO), and *R. solanacearum* (TYR) | *E. coli* | Glycerol | $33.9 \times 10^3$ | $0.848 \times 10^{-3}$ | Nakagawa *et al.* (2012) |
| Magnoflorine | *M. luteus* (MAO) and *C. japonica* (NCS, 6OMT, CNMT, 4′OMT and CYP80G2) | *E. coli and Saccharomyces cerevisiae* | Dopamine | $7.2 \times 10^3$ | 0.019 | Minami *et al.* (2008) |
| Scoulerine | *M. luteus* (MAO) and *C. japonica* (NCS, 6OMT, CNMT, 4′OMT and BBE) | *E. coli and S. cerevisiae* | Dopamine | $8.3 \times 10^3$ | 0.022 | Minami *et al.* (2008) |
| (*R*,*S*)-reticuline | *Papaver somniferum* (6OMT, CNMT and 4′OMT) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | $32.9 \times 10^3$ | 0.115 | Hawkins and Smolke (2008) |
| (S)-tetrahydrocolumbamine | *P. somniferum* (6OMT, CNMT, 4′OMT and BBE) and *Thalictrum flavum* (SMT) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | $60 \times 10^3$ | 0.052 | Hawkins and Smolke (2008) |
| (*S*)-tetrahydroberberine | *P. somniferum* (6OMT, CNMT, 4′OMT and BBE), *T. flavum* (SMT and CYP719A) and *Arabidopsis thaliana* (ATR1) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | $30 \times 10^3$ | 0.026 | Hawkins and Smolke (2008) |
| Salutaridine | *P. somniferum* (6OMT, CNMT, and 4′OMT) and *Homo sapiens* (CYP2D6 and CPR) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | $20 \times 10^3$ | 0.017 | Hawkins and Smolke (2008) |
| Stylopine | *A. thaliana* (ATR1), *Eschscholzia californica* (CFS and STS) *and P. somniferum* (6OMT, CNMT, 4OMT and BBE) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 676 | $1.176 \times 10^{-3}$ | Trenchard and Smolke (2015) |
| *cis-N*-methylstylopine | *A. thaliana* (ATR1), *E. californica* (CFS and STS) *and P. somniferum* (6OMT, CNMT, 4OMT, BBE and TNMT) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 548 | $0.954 \times 10^{-3}$ | Trenchard and Smolke (2015) |
| Protopine | *A. thaliana* (ATR1), *E. californica* (CFS and STS) *and P. somniferum* (6OMT, CNMT, 4OMT, BBE, TNMT and MSH) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 252 | $0.439 \times 10^{-3}$ | Trenchard and Smolke (2015) |
| Sanguinarine | *A. thaliana* (ATR1), *E. californica* (CFS, STS and P6H) *and P. somniferum* (6OMT, CNMT, 4OMT, BBE, TNMT and MSH) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 80 | $0.139 \times 10^{-3}$ | Trenchard and Smolke (2015) |
| (*R*,*S*)-reticuline | *P. somniferum* (6OMT, 4′OMT2 and CNMT) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 659 | 0.229 | Fossati *et al.* (2014) |

**Table 1** Continued

| Compound target | Source of plant gene inserted | Engineered host | Externally fed precursor | Titre (μg/L) | Yield (g product/g substrate) | References |
|---|---|---|---|---|---|---|
| (*S*)-stylopine | *E. californica* (P6H) *and P. somniferum* (6OMT, 4′OMT2, CNMT and P450R) | *S. cerevisiae* | (S)-scoulerine | 614 | 0.188 | Fossati *et al.* (2014) |
| Dihydrosanguinarine | *E. californica* (P6H) *and P. somniferum* (TNMT, MSH and P450R) | *S. cerevisiae* | (*S*)-stylopine | 1900 | 0.588 | Fossati *et al.* (2014) |
| Dihydrosanguinarine | *E. californica* (P6H) *and P. somniferum* (CFS, SPS, TNMT, MSH and P450R) | *S. cerevisiae* | (*S*)-scoulerine | 257 | 0.079 | Fossati *et al.* (2014) |
| Dihydrosanguinarine | *E. californica* (P6H) *and P. somniferum* (BBE, $BBE_{2\mu}$, CFS, SPS, TNMT, MSH and P450R) | *S. cerevisiae* | (*S*)-reticuline | 147 | 0.045 | Fossati *et al.* (2014) |
| Dihydrosanguinarine | *E. californica* (P6H) *and P. somniferum* (6OMT, CNMT, 4′OMT2, BBE, $BBE_{2\mu}$, CFS, SPS, TNMT, MSH and P450R) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 50 | 0.017 | Fossati *et al.* (2014) |
| Salutaridine | *P. somniferum* (SAS and CPR) | *S. cerevisiae* | (*R*)-Reticuline | 4911 | 0.149 | Fossati *et al.* (2015) |
| Thebaine | *P. somniferum* (SAS, CPR, SAR and SAT) | *S. cerevisiae* | (*R*)-Reticuline | 311 | $9.442 \times 10^{-3}$ | Fossati *et al.* (2015) |
| Thebaine | *P. somniferum* (SAS, CPR, SAR, SAT, CODM, T6ODM and CoR) | *S. cerevisiae* | Salutaridine | 311 | $9.500 \times 10^{-3}$ | Fossati *et al.* (2015) |
| Neopine | *P. somniferum* (SAS, CPR, SAR, SAT, CODM, T6ODM and CoR) | *S. cerevisiae* | Salutaridine | 9 | $0.275 \times 10^{-3}$ | Fossati *et al.* (2015) |
| Codeine | *P. somniferum* (SAS, CPR, SAR, SAT, CODM, T6ODM and CoR) | *S. cerevisiae* | (*R*)-Reticuline or Salutaridine | 12 or 19 | $0.364 \times 10^{-3}$ or $0.580 \times 10^{-3}$ | Fossati *et al.* (2015) |
| Morphine | *P. somniferum* (SAS, CPR, SAR, SAT, CODM, T6ODM and CoR) | *S. cerevisiae* | Codeine | 143 | $4.777 \times 10^{-3}$ | Fossati *et al.* (2015) |
| Codeine | *P. somniferum* (T6ODM and CoR) | *S. cerevisiae* | Thebaine | $7.7 \times 10^{3}$ | 0.025 | Thodey *et al.* (2014) |
| Morphine | *P. somniferum* (T6ODM, CoR and CODM) | *S. cerevisiae* | Thebaine | $4.7 \times 10^{3}$ | 0.015 | Thodey *et al.* (2014) |
| (S)-norcoclaurine | *Beta vulgaris* (CYP76AD1), *P. putida* (DODC) and *P. somniferum* (NCS) | *S. cerevisiae* | Glucose | 104.6 | $2.615 \times 10^{-6}$ | DeLoache *et al.* (2015) |
| (*S*)-reticuline | *B. vulgaris* (CYP76AD1), *P. putida* (DODC) and *P. somniferum* (NCS, 6OMT, CNMT, NMCH and 4OMT) | *S. cerevisiae* | Glucose | 80.6 | $2.015 \times 10^{-6}$ | DeLoache *et al.* (2015) |
| Norcoclaurine | *Rattus norvegicus* (PTPS, SepR, PCD, QDHPR and TyrH W166Y R37E R38E), *P. putida* (DODC) and *C. japonica* (NCS) | *S. cerevisiae* | Glucose | 0.56 | $0.028 \times 10^{-6}$ | Trenchard *et al.* (2015) |
| Reticuline | *Rattus norvegicus* (PTPS, SepR, PCD, QDHPR and TyrH W166Y R37E R38E), *P. putida* (DODC), *C. japonica* (NCS), *P. somniferum* (6OMT, CNMT, 4OMT and CPR) and *E. californica* (CYP80B1) | *S. cerevisiae* | Glucose | 19.2 | $0.960 \times 10^{-6}$ | Trenchard *et al.* (2015) |

 

**Table 1** Continued

| Compound target | Source of plant gene inserted | Engineered host | Externally fed precursor | Titre (μg/L) | Yield (g product/g substrate) | References |
|---|---|---|---|---|---|---|
| Thebaine | *Rattus norvegicus* (PTPS, SepR, PCD, QDHPR, TyrH W166Y R37E R38E and DHFR), *P. putida* (DODC), *C. japonica* (NCS), *P. somniferum* (6OMT, CNMT, 4OMT, CPR, SalAT and SalSyn), *E. californica* (NMCH) and *Papaver bracteatum* (SalR and DRS-DRR) | *S. cerevisiae* | (*R*,*S*)-Norlaudanosoline | 17 | $0.059 \times 10^{-3}$ | Galanie *et al.* (2015) |
| Thebaine | *Rattus norvegicus* (PTPS, SepR, PCD, QDHPR, TyrH W166Y R37E R38E and DHFR), *P. putida* (DODC), *C. japonica* (NCS), *P. somniferum* (6OMT, CNMT, 4OMT, CPR and SalAT), *E. californica* (NMCH), *P. bracteatum* (SalR and DRS-DRR) and a fusion enzyme CFS–SalSyn from *E. californica* (CFS) and *P. bracteatum* (SalSyn) | *S. cerevisiae* | Glucose | 6.4 | $0.320 \times 10^{-6}$ | Galanie *et al.* (2015) |
| Hydrocodone | *Rattus norvegicus* (PTPS, SepR, PCD, QDHPR, TyrH W166Y R37E R38E and DHFR), *P. putida* (DODC and morB), *C. japonica* (NCS), *P. somniferum* (6OMT, CNMT, 4OMT, CPR, SalAT and T6ODM), *E. californica* (NMCH), *P. bracteatum* (SalR and DRS-DRR) and a fusion enzyme CFS–SalSyn from *E. californica* (CFS) and *P. bracteatum* (SalSyn) | *S. cerevisiae* | Glucose | 0.3 | $0.015 \times 10^{-6}$ | Galanie *et al.* (2015) |

n.a., not available; MAO, monoamine oxidase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-O-methyltransferase; CNMT, coclaurine *N*-methyltransferase; 4OMT, 3-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase; $tyrA^{fbr}$, feedback-inhibition-resistant chorismate mutase/prephenate dehydrogenase; $aroG^{fbr}$, feedback-inhibition-resistant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; *tktA*, transketolase; *ppsA*, phosphoenolpyruvate synthetase; DODC, L-DOPA-specific decarboxylase; *TYR*, tyrosinase; CYP80G2, cytochrome P450 enzyme; BBE, berberine bridge enzyme; SMT, (*S*)-scoulerine 9-O-methyltransferase; CYP719A, canadine synthase, a cytochrome P450 enzyme; ATR1, *A. thaliana* P450 reductase; CYP2D6, human cytochrome P450; CPR, cytochrome P450 reductase; CFS, cheilanthifoline synthase; STS, stylopine synthase; P6H, protopine 6-hydroxylase; TNMT, tetrahydroprotoberberine cis-N-methyltransferase; MSH, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase; $BBE_{2\mu}$, BBE expression from a 2 μ vector; SPS, stylopine synthase; P450R, cytochrome P450 reductase; SAS, salutaridine synthase; SAT, salutaridinol acetyltransferase; T6ODM, thebaine 6-*O*-demethylase; CoR, codeinone reductase; CODM, codeine *O*-demethylase; CYP76AD1, tyrosine hydroxylase W13L F309L double mutant; NMCH, cytochrome P450 *N*-methylcoclaurine hydroxylase; PTPS, 6-pyruvoyltetrahydropterin synthase; SepR, sepiapterin reductase; PCD, pterin-4-alpha-carbinolamine dehydratase; QDHPR, quinonoid dihydropteridine reductase; TyrH W166Y R37E R38E, tyrosine hydroxylase triple mutant W166Y R37E R38E; GTPCHI, GTP cyclohydrolase I; CYP80B1, cytochrome P450 80B1; DHFR, dihydrofolate reductase; NMCH, N-methylcoclaurine hydroxylase; SalAT, salutaridinol 7-O-acetyltransferase; SalR, salutaridine reductase; SalSyn, salutaridine synthase; DRS-DRR, 1,2-dehydroreticuline synthase-1,2-dehydroreticuline reductase; morB, morphine reductase.

 

For cost-effective reasons, the commercial production of (*S*)-reticuline from central metabolites in *S. cerevisiae* is desired but has proven difficult. Although (*S*)-reticuline has been synthesized from fed (*R*,*S*)-norlaudanosoline in yeast (Fossati *et al.*, 2014, 2015; Hawkins and Smolke, 2008; Trenchard and Smolke, 2015), the upstream steps required for the synthesis of (*R*,*S*)-norlaudanosoline or the natural PIA scaffold (*S*)-norcoclaurine from tyrosine remained unknown (Figure 3). Recently, searches using fluorescent pigment betaxanthin sensor identified a plant tyrosine hydroxylase that was highly active in *S. cerevisiae* and using mutagenesis its activity was further improved (DeLoache *et al.*, 2015). The mutated tyrosine hydroxylase enabled the production of dopamine in yeast and, when coupled to subsequent *P. somniferum* biosynthetic genes, allowed for the synthesis of the PIA scaffold intermediates (*S*)-norcoclaurine and (*S*)-reticuline from glucose (DeLoache *et al.*, 2015). By connecting the central metabolism of yeast to the downstream steps of PIA biosynthesis, it will be possible to develop micro-organism capable of producing high-value PIAs at commercial scale. Recently, engineered yeast strains, containing modifications to divert greater carbon flux through tyrosine to (*S*)-reticuline, were used for the production of PIAs from sugar (Galanie *et al.*, 2015). The reticuline biosynthetic pathway was split into four genetic modules containing the nucleotide sequences of 17 biosynthetic enzymes allowing the production of thebaine and its conversion into codeine, oxycodone and hydrocodone (Galanie *et al.*, 2015).

For reticuline production, microbial platform seems more productive than 'old' platform. For example, in plants, a *P. somniferum* mutant, created to have increased reticuline content, produced up to 3%–4% of reticuline compared to trace amounts found in wild opium poppies (Fist *et al.*, 2005). However, the plant growth period (months) and the need of land/space for cultures can be limiting factors hindering commercial production. A knockdown of berberine bridge enzyme by RNAi in *E. californica* cells provided a plant cell line producing up to 6 mg of (*S*)-reticuline in a 20 mL medium in 2 weeks (Fujii *et al.*, 2007). Some of the limitations of this production are the need of high amount of inoculum, 1 g fresh weight of cells per 20 mL medium, coupled with the time needed for the growth and care of the inoculum. Microbial platform has the advantage of short time production and seems more easily up-scalable. For example, Nakagawa *et al.* (2011) engineered a strain of *E. coli* producing 46 mg/L in 60 h.

To date, there is no microbial platform for the production of PIAs of the phenethylisoquinoline, ipecac or Amaryllidaceae alkaloid groups due to the lack of molecular genetic tools. Integrating metabolome, transcriptome and proteome analyses and biochemical enzyme characterization will deliver many new genes and enzymes involved in pathways of PIA metabolism. To take full advantage of these genes and enzymes for synthetic biology and metabolic bioengineering, it will also be critical to understand the regulation of complex biosynthetic systems beyond the characterization of individual enzymes.

## Conclusion

Plants continue to be important producers of alkaloids. One key advantage of plants is that they naturally produce aromatic amino acid precursors and that they accomplish alkaloid biosynthesis powered by solar energy through photosynthesis, eliminating the need for supplying carbon precursors. Synthetic biology offers the sustainable mass production of PIAs. Several bacterial and yeast platforms have been developed, specifically for the production of benzylisoquinoline alkaloids, from which metabolic pathway and enzymes involved are known. There is still much work to be performed to render these platforms profitable commercially as they require the addition of expensive precursors and/or produce low concentration of targeted PIA.

Metabolic engineering of photoautotrophic organism, such as microalgae and cyanobacteria, may offer alternative systems for high-volume PIA synthesis (Gong *et al.*, 2011). As these micro-organisms can grow phototrophically, it can reduce the operation cost related to the growth media. In addition, the presence of chloroplasts in microalgae could not only be advantageous in allowing the reproduction of metabolic reaction and pathway normally occurring in plant plastids but also the sequestration of metabolites and enzymes could be used to increase the overall pathway efficiency. Ultimately, for micro-organism- or plant-based systems, scalability, ease of multigene combinatorial gene expression and cost-effective extraction procedures will be key factors for the commercially viable biotechnological production of PIAs. Also, a better understanding of alkaloid metabolic regulation at the transcriptional, cellular and biochemical levels is crucial to take advantage of new metabolic engineering technologies for improving the efficiency and sustainability of plant alkaloid production.

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## Conflict of interest

The authors confirm that this article content has no conflict of interests.

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